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Award Number: W81XWH-12-1-0571

TITLE: **Environment-Mediated Drug Resistance in Neuroblastoma**

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REPORT DATE: **October 2013**

TYPE OF REPORT: **Annual Report**

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE October 2013		2. REPORT TYPE Annual		3. DATES COVERED 30September2012 – 29September2013	
4. TITLE AND SUBTITLE Environment-Mediated Drug Resistance in Neuroblastoma				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-12-1-0571	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Yves A. DeClerck E-Mail: declerck@usc.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Children's Hospital Los Angeles 4650 Sunset Blvd., Los Angeles, CA 90027				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT During the past funded period, we have completed the first aim of our research project by demonstrating that interleukin-6 protects neuroblastoma cells from drug-induced apoptosis via activation of signal transduction and activator of transcription (STAT) 3 and that STAT3 is necessary to promote survival by upregulating Bcl-xL and survivin. In bone marrow samples of patients with neuroblastoma, we demonstrated the presence of a reciprocal loop of STAT3 activation in tumor cells and in monocytes-macrophages and T reg cells in which we found STAT-3 to be activated. We also demonstrated that monocytes cooperate with bone marrow-derived mesenchymal cells in activating STAT-3 by being a source of the agonistic soluble IL-6 receptor. This work was published in <i>Cancer Research</i> in July 2013. We have also generated NB-Tag mice that spontaneously develop neuroblastoma tumors in an IL-6 knock-out background. These mice develop tumors at a normal rate but we observed STAT3 activation in tumors suggesting the presence of alternate pathways than IL-6 responsible for STAT3 activation. We are currently testing whether these tumors are equally sensitive to chemotherapy. Further collaborative work with Dr. Seeger on the cooperation between MSC and monocytes indicate that in vivo, MSC enhance monocyte survival in tumors and increase the growth of neuroblastoma cells. Thus our data so far emphasize the important contribution of MSC and monocytes in neuroblastoma progression. They indicate that although IL-6 plays a role, there are alternate cytokines activating survival pathways such as STAT3. This will be the focus of our work during the second year of funding.					
15. SUBJECT TERMS Drug resistance, tumor microenvironment, neuroblastoma, JAK2, STAT3					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
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INTRODUCTION:

Neuroblastoma is the most common extra cranial solid tumor of childhood. Approximately 45% of children with neuroblastoma have aggressive tumors, nearly all of which are metastatic when diagnosed. This group includes patients with metastatic disease who are diagnosed at any age with MYCN-A tumors and patients older than 18 months of age with MYCN-non amplified (NA) tumors. During the past 20 years, long-term survival has steadily improved to 40% with increasing intensity of non-specific cytotoxic induction and consolidation therapy, followed by 13-cis-retinoic acid and anti-GD2 antibody immunotherapy of residual disease. It has become increasingly clear that tumor cells that may not be able intrinsically to resist therapeutic insults can acquire these properties as the result of specific interactions with the microenvironment. Although initially transient and reversible, this type of therapeutic resistance promotes the selection of surviving cells that have acquired permanent resistance. In 2005, the DeClerck laboratory identified the production of IL-6 by bone marrow mesenchymal stem cells as a major mechanism promoting osteolytic bone metastasis in neuroblastoma. In 2009 the laboratory demonstrated that in addition to promoting bone metastasis, IL-6 also promotes neuroblastoma cell survival and resistance to cytotoxic drugs. In collaboration, Drs. Seeger, Asgharzadeh and DeClerck demonstrated that not only MSC but also monocytes are a source of IL-6 in the tumor microenvironment of primary neuroblastoma tumors. In collaboration with Dr. Yu, partnering PI on this application, the DeClerck laboratory had obtained data demonstrating that STAT3 plays a pivotal role in IL-6-mediated drug resistance in neuroblastoma.

BODY-SCOPE OF WORK:

Task	Sp. Aim	Subtask	Performed by	Site
1. Cooperation between monocytes and tumor cells in IL-6/sIL-6R/STAT3-induced EMDR	1a	Drug sensitivity screen in co-cultures with monocytes	DeClerck/Seeger	CHLA
	1a	Effect of inhibitors of IL-6R/Jak2/STAT3 inhibitors	DeClerck/Seeger	CHLA
	1a	Analysis of survival and apoptotic proteins by Western blot, FACS	DeClerck/Seeger	CHLA
	1a	Co-cultures of fresh neuroblastoma cells-fresh bone marrow monocytes	Seeger	CHLA
2. Role of S1P on STAT3 activation and drug resistance	1b	Effect of IL-6, sIL-6R, and S1P on STAT3 activation, survival and drug resistance	Yu	COH
3. Determine the impact of S1PR1/JAK2/STAT3 signaling in monocytes to drug resistance	1c	Murine NBT2 neuroblastoma cells and human NB cells co-cultured with mouse and human monocytes in which S1PR1 is KO or KD	Yu/DeClerck	COH/CHLA
4. Determine whether S1PR1 and JKA2 are effective targets to block tumor cell-monocyte crosstalk	1d	Co-cultures of NB cells and monocytes in the presence of inhibitors of IL-6, JAK2 and S1PR1 and tested for drug resistance	Yu/DeClerck	CHO/CHLA
5. Effect of IL-6 in tumor and host cells on response to chemotherapy	2a	Breeding to obtain double transgenic homozygous IL-6 null	Asgharzadeh	CHLA
	2a	Imaging and monitoring for tumor development and tumor analysis by histology and TLDA microarrays	Asgharzadeh	CHLA
	2a	Treating NB-Tag mice and NB-Tag/IL-6 KO with cyclophosphamide and topotecan.	Asgharzadeh	CHLA
6. Contribution of bone marrow-derived IL-6 to response to therapy	2b	Transplantation of NB-Tag IL-6 -/- mice with WT bone marrow	Yu/Asgharzadeh	COH/CHLA
	2b	Transplantation into NB-Tag mice, treatment with Poly I:C and monitoring of tumor development	Yu/Asgharzadeh	COH/CHLA
	2b	Effect on drug response: mice will be treated with cyclophosphamide/topotecan and monitored for response	Yu/Asgharzadeh	COH/CHLA

7. Develop strategies that can be translated into clinical trials to overcome EMDR	3a	Testing tocilizumab in SCID mice implanted with human NB cells and monocytes	DeClerck/Seeger	CHLA
	3a	Testing AZD 1480 in SCID mice implanted with human NB cells and monocytes	DeClerck/Seeger/Yu	CHLA/COH

KEY RESEARCH ACCOMPLISHMENTS:

Task 1. Cooperation between monocytes and tumor cells in IL-6/sIL-6R/STAT3-induced EMDR: This task has been completed (Ara *et al.* 2013). Consistent with NB cells not producing IL-6 (Ara *et al.* 2009), we determined that STAT3 was not constitutively active in most neuroblastoma cell lines but was rapidly activated upon treatment with IL-6 alone and in combination with sIL-6R. Treatment of neuroblastoma cells with IL-6 protected them from drug-induced apoptosis in a STAT3-dependent manner because the protective effect of IL-6 was abrogated in the presence of a STAT3 inhibitor and upon STAT3 knockdown. STAT3 was necessary for the upregulation of several survival factors such as survivin and Bcl-xL when cells were exposed to IL-6. In collaboration with Dr. Seeger's group, we then demonstrated that IL-6-mediated STAT3 activation was enhanced by sIL-6R produced by human monocytes, pointing to an important function of monocytes in promoting IL-6-mediated EMDR. Our data also point to the presence of reciprocal activation of STAT3 between tumor cells and bone marrow stromal cells, including not only monocytes but also regulatory T cells (Treg) and non-myeloid stromal cells.

Task 2. Role of S1P on STAT3 activation and drug resistance: This task has been performed by our collaborator at City of Hope, Dr. Hua Yu (see separate progress report from her). She showed that endogenous and IL-6/S1P-induced JAK2-STAT3 signaling in human neuroblastoma is associated with growth suppression and apoptosis. In order to understand the mechanism of drug resistance and to develop targeted strategies for combating drug resistance, she has generated etoposide-resistant human neuroblastoma cells. And demonstrated that etoposide-resistant human cells have highly elevated S1PR1 expression and STAT3 activity.

Task 3. Determine the impact of S1PR1/JAK2/STAT3 signaling in monocytes to drug resistance: This task has not been initiated yet as we are presently working on generating the conditional knock out.

Task 4. Determine whether S1PR1 and JAK2 are effective targets to block tumor cell-monocyte crosstalk. We have begun to test the effect of STAT3 blocking on drug resistance induced in co-cultures of human monocytes and mesenchymal cells. We have used 2 inhibitors of JAK2 (AZD4188 and Ruxolitinib). Preliminary data (see Figure 1).

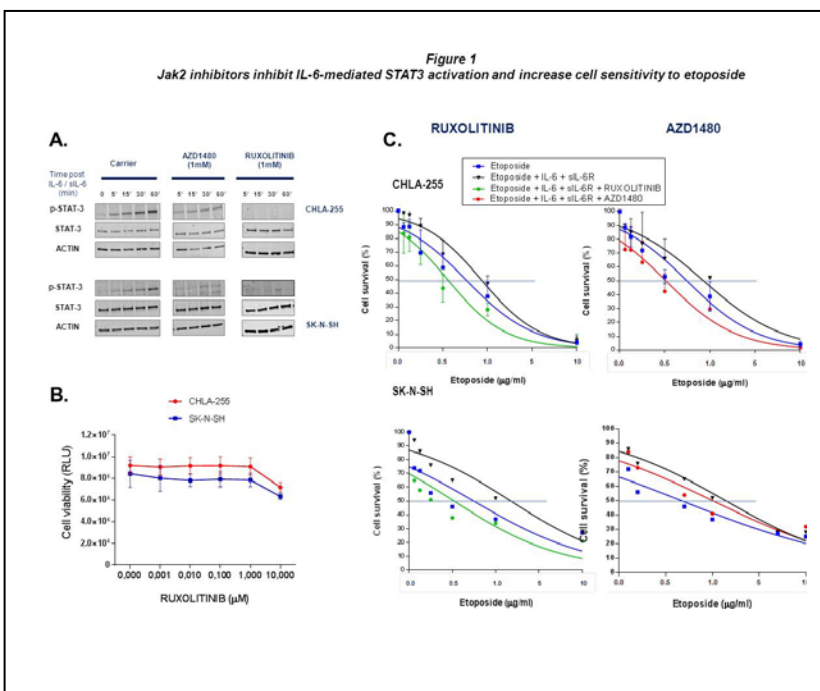


Figure 1. Jak2 inhibition restores the sensitivity of NB cells to etoposide in the presence of IL-6 and sIL-6R. A. CHLA-255 and SK-N-SH cells were pre-treated with ruxolitinib or AZD-418 prior to being treated with IL-6 and sIL-6R. Cells were harvested and lysates examined for STAT3 and pSTAT3 by Western blot at indicated times. B. NB cells were incubated in the presence of indicated concentrations of ruxolitinib for 48 h. before being examined for viability by CytoGlow assay. The data represent the mean (\pm SD) luminescence (in RLU) of triplicate samples. C. Cells treated as above were incubated in the presence of etoposide at indicated concentrations and examined for viability after 48 h. The data represent the mean luminescence (\pm SD) of triplicate samples.

We demonstrate that these inhibitors are effective in blocking STAT3 activation in cells exposed to IL-6 and sIL-6R and increase the sensitivity of neuroblastoma cells to

etoposide. However when tested for etoposide sensitivity in the presence of conditioned medium from

mesenchymal cells, ruxolitinib is not effective in increasing sensitivity. This suggests the presence of by-pass alternate pathways. We have obtained preliminary evidence (Figure 2) that upon exposure to the conditioned medium of MSC (and not human skin fibroblasts), not only STAT3 but also Erk1/2 and Akt become activated. In our planned experiments, we will test the effect of a combination of Erk1/2, S1PR1 and STAT3 inhibitors.

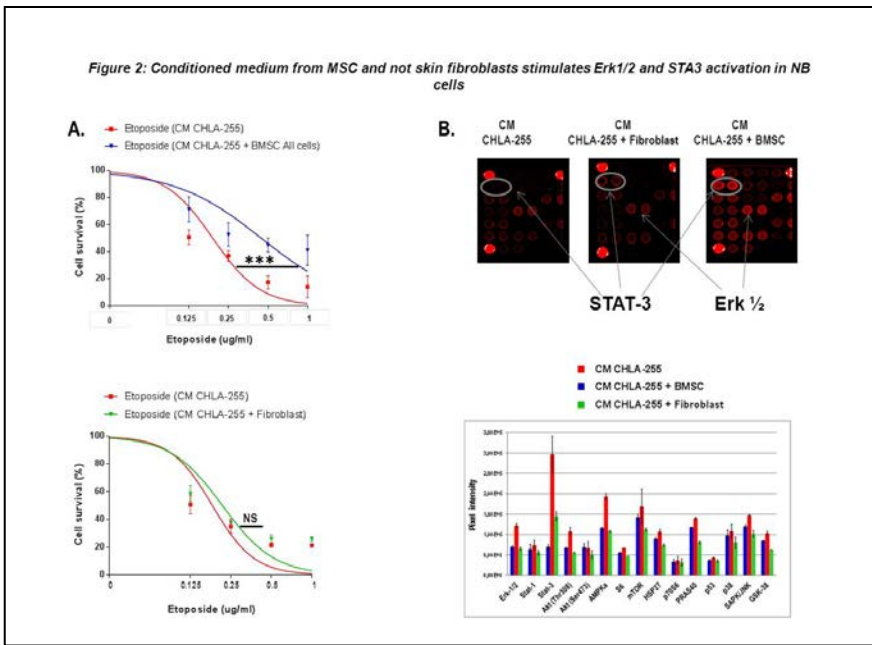
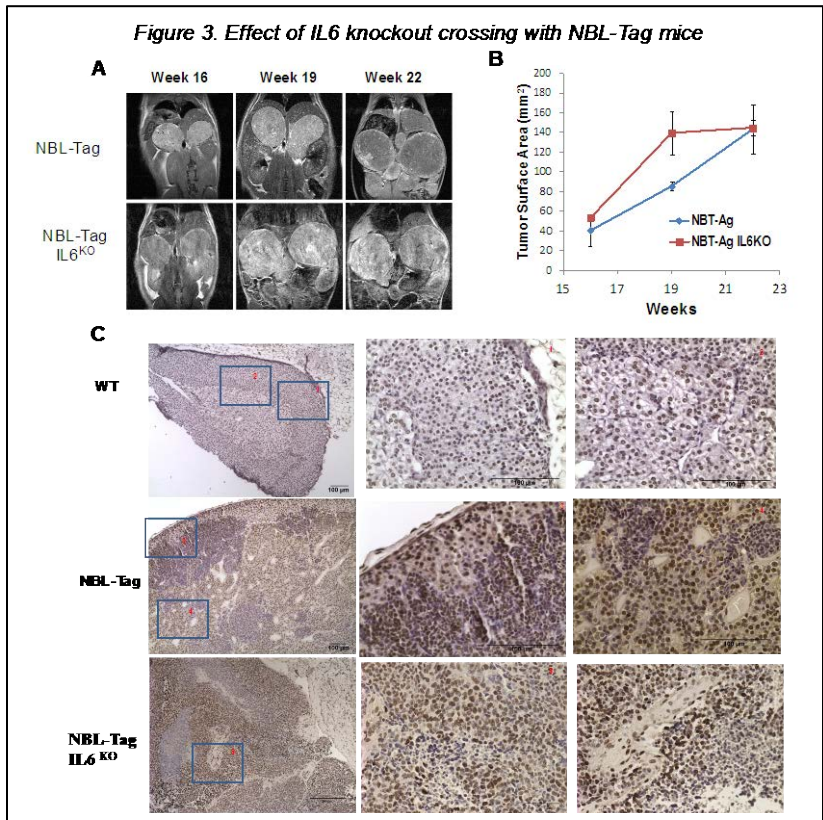


Figure 2: Conditioned medium from MSC but not skin fibroblasts increases NB cells resistance to etoposide and activates Erk1/2 and STAT3. A. CHLA-255 NB cells were tested for their sensitivity to etoposide as described in Fig. 1. Cells were preincubated in the presence of conditioned medium of CHLA-255 cells co-cultures with human MSC (top) or human skin fibroblasts (bottom). B. CHLA-255 cells were exposed to their own medium (control) of conditioned medium from human fibroblasts or MSC. After 30 min. cells were harvested, lysed and examined for the presence of phosphoproteins by dot blot analysis. The bottom graph represents the signal intensity reading for each phosphoprotein.

Task 5. Effect of IL-6 in tumor and host cells on response to chemotherapy: Our collaborator Dr. Asgharzadeh has generated NB-Tag mice in an IL-6 KO background. He found that NB tumors in these mice develop at the anticipated rate seen in WT Tag mice, indicating that lack of IL-6 does not affect tumor initiation and growth (Figure 3). He is now completing experiments testing the response of these tumors to chemotherapy. Interestingly, an analysis of these tumors for pSTAT3 by Western blot and



immunohistochemistry revealed the presence of pSTAT3, suggesting the presence of alternate pathways of activation.

Figure 3: Effect of IL6 knockout crossing with NB-Tag mice. A. MRI of abdominal cavity of representative mice from NB-Tag and NB-TagxIL6^{KO} at 3 different ages. B. There were no difference between growth of tumors between the two groups (n=3-4 mice per group). C. Immunohistochemical analysis of adrenal gland reveal no pSTAT3 expression while both NB-Tag and NB-TagxIL6^{KO} express pSTAT3 suggesting alternative pathways of activation independent of IL6 production.

Task 6: Contribution of bone marrow-derived IL-6 to response to therapy: We have not yet performed these experiments as we are waiting to analyze the response of tumors in NBTag/IL-6 KO mice.

Task 7: Develop strategies that can be translated into clinical trials to overcome EMDR: We have tested the effect of tocilizumab in mice implanted with human NB tumor cells and human monocytes. These experiments have not been conclusive and

although they initially showed some effects (Figure 4), repeated experiments failed to show a statistically significant effect.

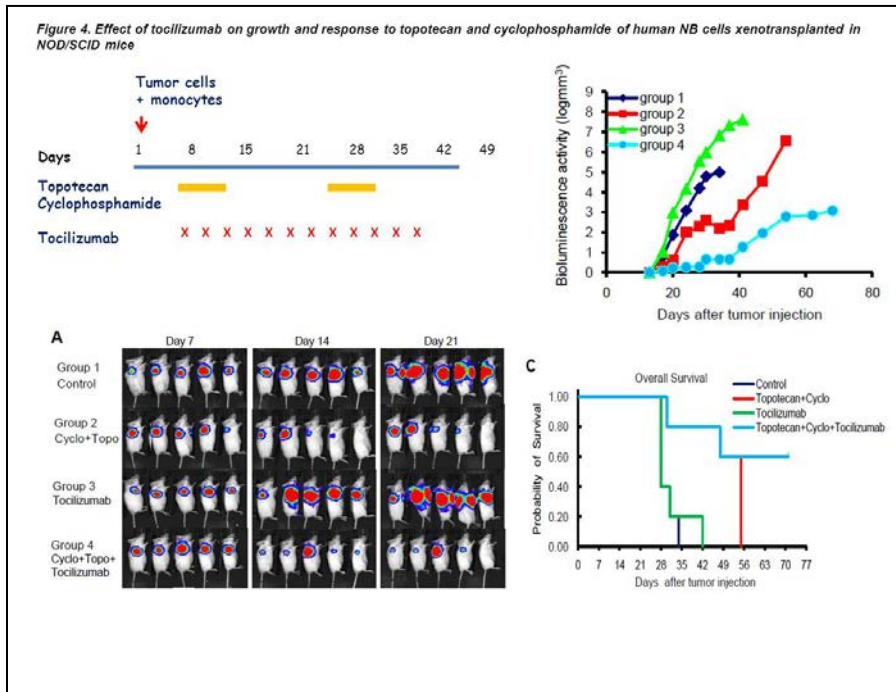


Figure 4: NOD/SCID mice ($n=5$ in each group) were xenotransplanted with CHLA-255 Luc cells and human monocytes and treated as per the schedule shown in left upper corner. **A.** Luminescence images obtained at day 7, 14 and 21. **B.** Quantitative luminescence measurements obtained over time. **C.** Kaplan-Meier survival curve.

These data, together with the results of our IL-6KO experiments (task 5) suggest that although IL-6 contributes to NB growth and drug resistance, there is more than this pathway. We are currently redirecting our experiments to test a combination of agents that will target not only STAT3 but also Erk1/2 and Akt, and to identify other soluble factors produced by MSC and monocytes involved in drug resistance.

REPORTABLE OUTCOME:

Yang, F., Jove, V., Buettner, R., Xin, H., Wu, J., Wang, Y., Nam, S., Xu, Y., Ara, T., DeClerck, Y.A., Seeger, R., Yu, H. and Jove, R. Sorafenib inhibits endogenous and IL-6/S1P induced JAK2-STAT3 signaling in human neuroblastoma, associated with growth suppression and apoptosis. *Cancer Biol Ther*, 13:534-541, 2012.

Ara, T., Nakata, R., Shimada, H., Buettner, R., Groshen, S.G., Ji, L., Sheard, M., Yu, H., Jove, R., Seeger, R.C. and DeClerck, Y.A. Critical role of STAT3 in interleukin-6-mediated drug resistance in human neuroblastoma. *Cancer Res*, 73:3852-3864, 2013.

CONCLUSIONS:

Over the last year we have generated data that brought additional light to the mechanism by which the TEM protects tumor cells from drug-induced apoptosis. In particular we show:

1. That MSC and monocytes collaborate in part by producing IL-6 and sIL-2R that activate STAT3 in tumor cells and promote survival.
2. We became increasingly aware that IL-6/sIL-6R/STAT3 is not the only pathway responsive for drug resistance and that other cytokines/growth factors and other pathways could contribute. We are thus exploring a combination of pathway inhibitors for their ability to restore drug sensitivity in the presence of CM from MSC.

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Ara, T., Nakata, R., Shimada, H., Buettner, R., Groshen, S.G., Ji, L., Sheard, M., Yu, H., Jove, R., Seeger, R.C. and DeClerck, Y.A. Critical role of STAT3 in interleukin-6-mediated drug resistance in human neuroblastoma. *Cancer Res*, 73:3852-3864, 2013.

APPENDICES:

Ara *et al.*, *Cancer Research* 2013



Cancer Research

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Cancer Res 2013;73:3852-3864. Published OnlineFirst April 30, 2013.

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Critical Role of STAT3 in IL-6–Mediated Drug Resistance in Human Neuroblastoma

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Abstract

Drug resistance is a major cause of treatment failure in cancer. Here, we have evaluated the role of STAT3 in environment-mediated drug resistance (EMDR) in human neuroblastoma. We determined that STAT3 was not constitutively active in most neuroblastoma cell lines but was rapidly activated upon treatment with interleukin (IL)-6 alone and in combination with the soluble IL-6 receptor (sIL-6R). Treatment of neuroblastoma cells with IL-6 protected them from drug-induced apoptosis in a STAT3-dependent manner because the protective effect of IL-6 was abrogated in the presence of a STAT3 inhibitor and upon STAT3 knockdown. STAT3 was necessary for the upregulation of several survival factors such as survivin (BIRC5) and Bcl-xL (BCL2L1) when cells were exposed to IL-6. Importantly, IL-6–mediated STAT3 activation was enhanced by sIL-6R produced by human monocytes, pointing to an important function of monocytes in promoting IL-6–mediated EMDR. Our data also point to the presence of reciprocal activation of STAT3 between tumor cells and bone marrow stromal cells including not only monocytes but also regulatory T cells (Treg) and nonmyeloid stromal cells. Thus, the data identify an IL-6/sIL-6R/STAT3 interactive pathway between neuroblastoma cells and their microenvironment that contributes to drug resistance. *Cancer Res*; 73(13); 3852–64. ©2013 AACR.

Introduction

Over the last 10 years, it has become increasingly appreciated that tumor cells that lack the intrinsic ability to initiate an angiogenic response, to resist the injury of therapies or to metastasize, can acquire such properties through the influence of the microenvironment (1). The acquisition of these properties occurs through complex interactions between tumor cells and a variety of stromal cells such as carcinoma-associated fibroblasts, endothelial cells, adipocytes, myofibroblasts, mesenchymal cells, and innate and adaptive immune cells (2–5). The identification of pathways involved in these interactions has therefore been the subject of intensive investigation over the recent years with the anticipation that these pathways will be novel targets for anticancer therapy (6). Among the characteristics that tumor cells acquire through their interaction with the microenvironment is drug resistance, a major cause of failure to eradicate cancer. The acquisition of drug

resistance through interactions between tumor cells and their environment, known as "environment-mediated drug resistance" (EMDR; ref. 7), is an important contributor to the emergence of minimal residual disease in cancer. EMDR occurs through complex adhesion-dependent and -independent interactions between tumor cells and the extracellular matrix (ECM) and stromal cells (8, 9). The bone marrow microenvironment plays a particularly important role in EMDR as it is an abundant source of ECM proteins, cytokines, and growth factors produced by mesenchymal and hematopoietic stem cells and their progeny that promote homing and survival (10).

The bone marrow is also the most frequent site of metastasis in neuroblastoma, a tumor derived from the neural crest that is the second most common solid malignancy affecting children (11, 12). It is a source of multiple chemokines, cytokines, and growth factors including interleukin (IL)-6. We have previously shown that in the case of neuroblastoma, IL-6 is not produced by tumor cells but by bone marrow-derived mesenchymal stem cells (BMMSC) and tumor-associated macrophages (TAM; refs. 13–15). The paracrine production of IL-6 by BMMSC plays a dual role in neuroblastoma bone marrow and bone metastasis. It activates osteoclasts, promoting the formation of osteolytic lesions and stimulates the growth and survival of neuroblastoma cells (16). Among the signaling pathways activated by IL-6, is the signal transducer and activator of transcription (STAT3) that plays a central role in the communication between tumor cells and immune cells (17). STAT3 was initially discovered as a transcription factor induced by IFN- γ (18). It is considered an oncogene as it is required for the oncogenic transformation activity of v-Src (19). It has multiple protumorigenic functions including the

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Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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doi: 10.1158/0008-5472.CAN-12-2353

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promotion of tumor cell proliferation, survival, invasion, metastasis, and angiogenesis (20–22). In addition, STAT3 is a major contributor to inflammation (23) and has been shown to promote the acquisition of chemo- and radioresistance (24, 25). In most cancers STAT3 is constitutively active, but its activation can also occur through the influences of the micro-environment and in particular IL-6 (26). IL-6 binds to a heterodimeric receptor made of 2 subunits, the gp80 α subunit (IL-6R) that is the ligand-binding unit and the gp130 β subunit that is the signal-transducing unit, which via phosphorylation of Janus-activated kinases (JAK), activates STAT3 (27). The gp80 unit is also present in a soluble form [soluble IL-6 receptor (sIL-6R)] that has an agonistic effect through its trans signaling function (28). Here, we have explored the role of STAT3 activation by IL-6 and sIL-6R in EMDR in human neuroblastoma.

Materials and Methods

Cell culture

Human neuroblastoma cell lines were cultured as previously reported (16). The cells were authenticated by genotype analysis using AmpFISTR Identifier PCR kit and GeneMapper ID v. 3.2 (Applied Biosystems). Human BMMSC were purchased from AllCells LLC. Monocytes of normal healthy donors were obtained from peripheral blood and separated by Ficoll density gradient centrifugation using a human monocyte isolation kit (Miltenyi Biotec).

Reagents

Rabbit polyclonal antibodies against pY⁷⁰⁵ STAT3, STAT3, survivin, Bcl-xL XIAP, Bcl-2, Mcl-1, uncleaved and cleaved caspase-3 and -9, and cytochrome C and Alexa Fluor 488–conjugated antibodies against survivin and Bcl-xL were purchased from Cell Signaling Technology, Inc. A rabbit polyclonal antibody against actin and a mouse monoclonal antibody (mAb) against β -actin were purchased from Sigma-Aldrich. A mouse polyclonal antibody against STAT3 was purchased from Cell Signaling Technology, Inc. The following secondary antibodies were used for Western blot analyses, immunocyto-fluorescence, and immunohistochemistry: biotinylated anti-rabbit immunoglobulin G (IgG; H+L; Vector Labs), donkey anti-rabbit IRDye 800CW, donkey anti-mouse IRDye 680 (LI-COR Biosciences), goat horseradish peroxidase (HRP)–conjugated anti-rabbit Streptavidin Dylight 488 (Jackson ImmunoResearch), goat anti-mouse Alexa Fluor 555 (Invitrogen), and donkey anti-rabbit IgG (Thermo Scientific). Antibodies against the following markers were from BD Biosciences: CD14-V450, phospho-Stat3 (pY705)-PE, CD4-APC-H7, CD25-PE-Cy7, FoxP3-PerCP-Cy5.5, and GD2-APC. Anti-CD3-Alexa Fluor 488 was from BioLegend. Anti-CD163-Alexa Fluor 700 was from R&D Systems. Anti-CD45-Krome Orange was from Life Technologies. Human FcR blocking agent was from Miltenyi Biotec. BD Fix I Buffer and BD Perm III Buffer were from BD Biosciences. Etoposide (Ben Venue Laboratories, Inc.) and melphalan (Sigma-Aldrich) were dissolved in acidified-ethanol at a stock concentration of 64 μ g/mL. The STAT3 inhibitor stattic (29) was purchased from Calbiochem and solubilized in dimethyl sulfoxide (DMSO) at a stock concentration of

60 mmol/L. Recombinant human IL-6 and sIL-6R were purchased from R&D Systems. A humanized mouse monoclonal function-blocking antibody against IL-6R (tocilizumab; ref. 30) was purchased from Genentech, Inc.

Western blot analysis

Western blot analyses were conducted as previously described (16). Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer supplemented with 1 tablet of complete mini-EDTA protease inhibitor cocktail (Roche Diagnostics) or halt protease and phosphatase inhibitor cocktail (Thermo Scientific). The detection of immune complexes and their quantification was conducted using either the Odyssey Infrared Imaging Systems (LI-COR Biosciences) or chemiluminescence with an HRP antibody detection kit (Denville) and the NIH ImageJ software for analysis.

Cell viability assay

Cell viability in the presence of cytotoxic drugs was determined by fluorescence-based cytotoxicity assay using digital imaging microscopy (DIMSCAN; ref. 31).

Flow cytometry

For JC-1 stain, cultured cells were detached in cell dissociation buffer (Invitrogen) and stained for 30 minutes in the presence of JC-1 dye (10 mg/mL; MitoProbe JC-1 Assay Kit; Invitrogen) before being analyzed by flow cytometry. For Annexin V stain, cultured cells were resuspended in 1 \times Annexin V-binding buffer. Annexin V and propidium iodide (PI) staining were conducted using an Annexin V–fluorescein isothiocyanate (FITC) apoptosis detection kit II according to the manufacturer's instructions (BD Pharmingen).

Enzyme-linked immunosorbent assay (ELISA)

The levels of human IL-6 and sIL-6R in serum-free conditioned medium of cultured cells were determined by ELISA using the Quantikine Immunoassay Kit or DuoSet ELISA Development Kit from R&D Systems.

siRNA-based gene knockdown

Downregulation of the expression of STAT3 was done using the Signal Silence STAT3 siRNA Kit (Cell Signaling Technology, Inc.). Cells plated in 6-well plates (2.5×10^5 cells) were transfected with scrambled siRNA or STAT3 siRNA and a fluorescein-conjugated siRNA (to verify transfection efficiency) using the Lipofectamine RNAi MAX reagent (Invitrogen). The downregulation of the protein (STAT3) was verified by Western blot analysis on cell lysates obtained 72 hours after transfection. The following siRNA sequences were used from SignalSilence: STAT3 siRNA1, cat. no. 6582 and STAT3 siRNA I, cat. no. 6580.

Immunohistochemistry and immunofluorescence

Paraffin-embedded sections (4 μ m) of bone marrow biopsies were obtained through the Children's Oncology Group Biorepository by H.S. These samples were acquired after informed consent was obtained and upon approval of Children's Hospital Los Angeles (CHLA) Institutional Review Board. Antigen

unmasking was conducted by proteinase K treatment (20 µg/mL for 10 minutes at 25°C). The slides were incubated overnight at 4°C in the presence of the following primary antibodies: a rabbit anti-human pY705 STAT3, survivin, or Bcl-xL, a mouse anti-CD68 or a rabbit antityrosine hydroxylase mAb (dilutions 1:100). After washing 3× with 0.1% Triton-X 100 in PBS, the slides were incubated in the presence of one of the following secondary antibodies: an Alexa Fluor 488-conjugated goat anti-mouse IgG or anti-mouse IgG antibody (dilution 1:50) for 1 hour at room temperature. For immunofluorescence, slides were mounted in 4',6-diamidino-2-phenylindole (DAPI) containing Vectashield medium. For dual immunohistochemistry, the Bond Polymer Refine Detection (Leica Biosystems Newcastle Ltd.) was used. Slides were heated at pH 6 for 20 minutes before being processed. The biotin-free polymeric HRP linker (DS 9800) was used for the detection of pSTAT3 and the biotin-free polymeric alkaline phosphatase linker (DS 9390) was used for the detection of Protein Gene Product (PGP) 9.5 and CD45. As primary antibodies, we used a mouse anti-human PGP 9.5 from Leica (PA0286) undiluted, a mouse anti-CD45 mAb from Abcam (ab8216) at a 1:25 dilution, and a rabbit anti-pSTAT3 (Tyr705) polyclonal antibody from Cell Signaling Technology, Inc. (9131) at a 1:25 dilution. Primary and secondary antibodies were incubated for 30 minutes.

Immunocytofluorescence

Cells were cultured in Lab-Tek II 8 chamber slides for 48 hours (2×10^4 and 10×10^4 cells/well). Cells were then washed, treated with IL-6 and sIL-6R for 30 minutes, and then fixed with 4% formaldehyde in PBS for 10 minutes and permeabilized with 0.1% Triton-X100 in 15% FBS in PBS for 5 minutes, before being incubated in the presence of an anti-human pSTAT3 or STAT3 antibody overnight at 4°C.

Statistical analysis

For the analyses of cell viability, the luciferase or fluorescence activity readings were assumed to have a lognormal distribution and were transformed to the \log_{10} scale before analyses were conducted. ANOVA was used to examine the differences in mean cell viability among groups and the Student (two-tailed) test was used to compare 2 groups in the apoptotic assays. All *P* values reported were two-sided. A *P* value of less than 0.05 was considered significant. Data were analyzed with software STATA version 11.2 (StataCorp LP).

Results

IL-6 and sIL-6R activate STAT3 in neuroblastoma cells

We had previously reported that with a few exceptions most human neuroblastoma cells do not produce significant amounts of IL-6 and do not secrete sIL-6R, but express the 2 IL-6R subunits (16). To determine the status of STAT3 activation in neuroblastoma, we initially examined 8 human tumor cell lines for the expression of STAT3 and phospho Y⁷⁰⁵ STAT3 (pSTAT3) by Western blot analysis under baseline conditions and upon treatment with IL-6, sIL-6R, and their combination. This analysis revealed a low amount of pSTAT3 in most untreated cells (except in SK-N-SH and CHLA-90 cells), indicating a general absence of constitutive activation of

STAT3 in neuroblastoma (Fig. 1A). However, when cells were treated with IL-6 alone and in particular in combination with sIL-6R, we observed a significant increase in pSTAT3 after 30 minutes in all cell lines. Activation of STAT3 in CHLA-255 and CHLA-90 cells was confirmed by immunocytofluorescence (Fig. 1B). This analysis revealed the presence of cytoplasmic STAT3 in both cell lines. In CHLA-255 cells, pSTAT3 was not detected in the absence of IL-6 but became detectable in the nucleus upon treatment with IL-6 alone or in combination with sIL-6R. In contrast, nuclear pSTAT3 was detected in CHLA-90 cells with or without treatment with IL-6 and sIL-6R. To confirm the role of IL-6 in STAT3 activation, we showed that incubation of CHLA-255 cells with a mAb against human IL-6R (tocilizumab) before treatment with IL-6 and IL-6 plus sIL-6R suppressed STAT3 phosphorylation (Supplementary Fig. S1A) and the binding of STAT3 to DNA as determined by electrophoretic mobility shift assay (EMSA; Supplementary Fig. S1B). We also showed that CHLA-255 cells transiently transfected with a STAT3 responsive promoter construct driving the firefly luciferase reporter gene (*STAT3Luc*) and a renilla luciferase vector had a 4-fold increase in firefly/*Renilla* luciferase activity when treated with IL-6 plus sIL-6R, and that this increase in activity was suppressed in the presence of tocilizumab (Supplementary Fig. S1C). Thus, altogether the data showed that IL-6 is an effective and specific activator of STAT3 in human neuroblastoma, in particular in the presence of sIL-6R.

IL-6 and sIL-6R protect neuroblastoma cells from drug-induced apoptosis

To show the role of IL-6 in chemoresistance, we selected 2 drug-sensitive neuroblastoma cell lines (CHLA-255 and SK-N-SH) and tested the effect of IL-6 and sIL-6R on their survival in the presence of etoposide and melphalan (32, 33). This analysis revealed a dose-dependent decrease in the survival fraction in both cell lines upon exposure to etoposide or melphalan for 24 hours (Fig. 2A–D). However, when cells were pretreated with IL-6 (alone and with sIL-6R) and then exposed to the drug, we observed a significant increase in the survival fraction when compared with the drug alone. As anticipated, the addition of sIL-6R alone in the absence of IL-6 failed to protect neuroblastoma cells from etoposide-induced apoptosis (Supplementary Fig. S3A). We next examined the effect of IL-6 and sIL-6R pretreatment on mitochondrial membrane depolarization and caspase-3 and -9 activation in cells treated with etoposide or melphalan (Fig. 3). The data showed that pretreatment of CHLA-255 cells with IL-6 alone or with sIL-6R resulted in less mitochondrial membrane depolarization (Fig. 3A and B). IL-6 also inhibited the cytoplasmic release of cytochrome C upon treatment with etoposide (Fig. 3C). Consistently, pretreatment of CHLA-255 cells with IL-6 inhibited the cleavage of caspase-3 and -9 in the presence of increased concentrations of etoposide or melphalan (Fig. 3D and E). Altogether, the data thus indicated that IL-6 had a protective effect on drug-induced intrinsic apoptosis.

STAT3 is necessary for IL-6-mediated drug resistance

We next asked the question whether STAT3 activation was necessary for the protective effect of IL-6 on drug-induced

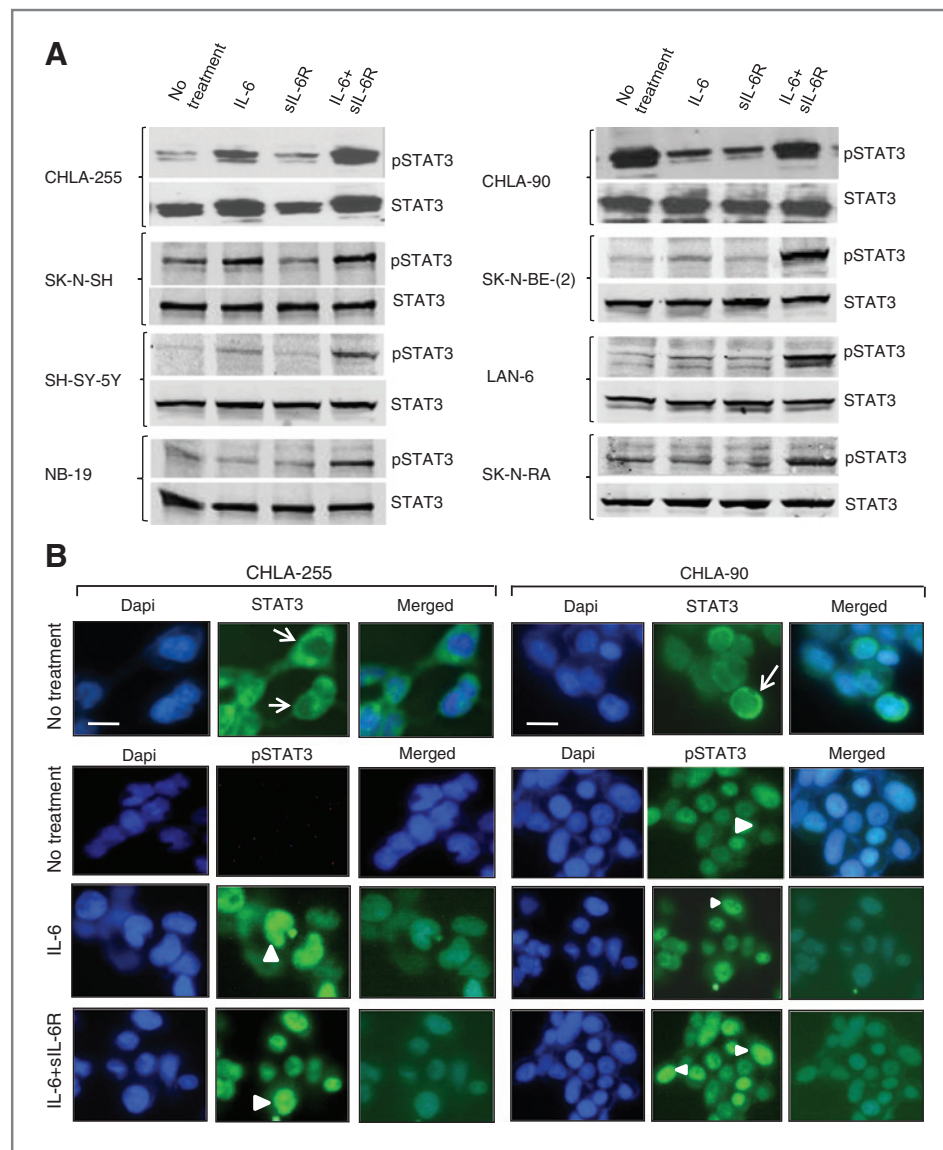


Figure 1. IL-6 and siL-6R activate STAT3 in human neuroblastoma cells. **A**, the presence of phosphorylated pY⁷⁰⁵ STAT3 and STAT3 was detected by Western blot analysis in total cell lysates from 8 neuroblastoma cell lines. When indicated, cells were treated with IL-6 (10 ng/mL) and siL-6R (25 ng/mL). Lysates were obtained 30 minutes after treatment. The data are representative of 2 to 3 separate experiments showing similar results. **B**, the presence of STAT3 and pY⁷⁰⁵ STAT3 in cultured CHLA-255 (left) and CHLA-90 (right) cells untreated or treated for 30 minutes with IL-6 alone or with siL-6R as described earlier was examined by immunocytofluorescence. Arrow, cytoplasmic STAT3. Arrowhead, nuclear pSTAT3 (scale bar, 20 μ m).

apoptosis. We first used stattic, a small-molecule inhibitor of STAT3 activation (29) and showed that treatment of CHLA-255 cells with stattic (0.5 to 20 μ M/L) prevented STAT3 activation by IL-6 plus siL-6R (Fig. 4A). We then examined the effect of stattic (at 2.5 μ M/L, to maintain 80% cell viability) on apoptosis induced by etoposide in CHLA-255 cells pretreated with IL-6 and siL-6R. The data indicated that stattic restored etoposide-induced apoptosis in the presence of IL-6 and IL-6 plus siL-6R to levels observed in the absence of IL-6 and stattic (Fig. 4B).

The necessary role of STAT3 in IL-6-mediated drug resistance was confirmed by examining the effect of

siRNA-mediated STAT3 knockdown on neuroblastoma cell sensitivity to etoposide in the presence of IL-6 and siL-6R. The data indicated that transfection of CHLA-255 cells with a combination of 2 siRNA sequences resulted in an 80% STAT3 knockdown and a complete suppression of STAT3 activation upon treatment with IL-6 alone or with siL-6R when compared with a scramble siRNA sequence (Fig. 4C). STAT3 knockdown in CHLA-255 cells restored their sensitivity to etoposide in the presence of IL-6 and siL-6R to levels close to the level of apoptosis observed in the absence of IL-6 (Fig. 4D). The knockdown of STAT3 in SK-N-SH cells had a similar effect on the sensitivity of these cells to etoposide in

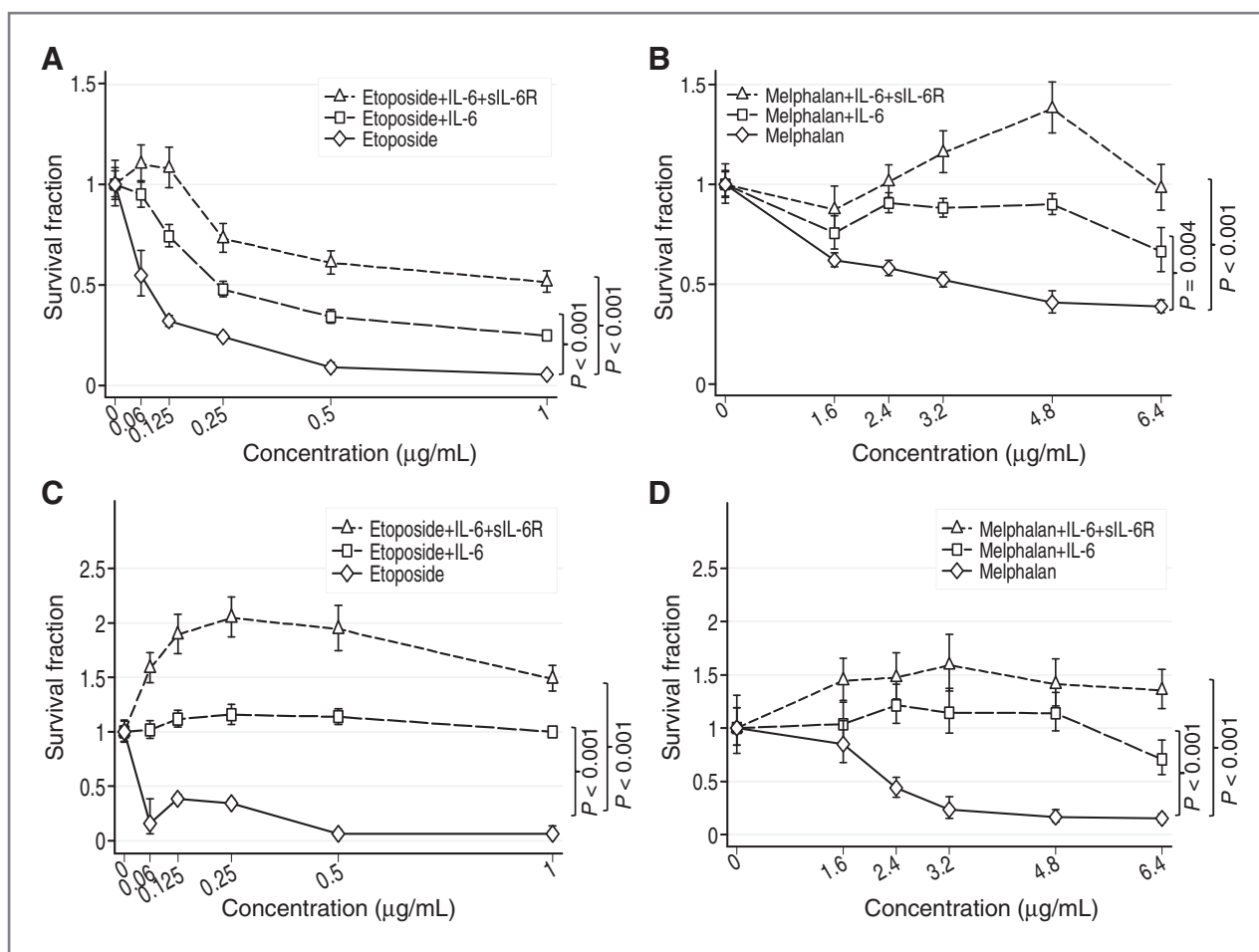


Figure 2. IL-6 and sIL-6R protect neuroblastoma cells from drug toxicity. CHLA-255 (A and B) and SK-N-SH cells (C and D) were treated with IL-6 (10 ng/mL) alone or with sIL-6R (25 ng/mL) for 24 hours before being exposed to etoposide (A and C) or melphalan (B and D) at indicated concentrations. Cell viability was determined after 24 hours of drug exposure by DIMSCAN analysis. The data represent the mean (\pm SD) survival fraction with a minimum of 10 replicates for each experimental condition.

the presence of IL-6 and sIL-6R (Supplementary Fig. S2A and S2B). The data thus showed that the protective effect of IL-6 on drug-induced apoptosis in neuroblastoma was STAT3 activation-dependent.

Upregulation of survival proteins by IL-6 is STAT3 dependent

We investigated the effect of IL-6 on the expression of survival proteins in CHLA-255 cells. The data indicated an increase in the expression of survivin, Mcl-1, XIAP-1, and Bcl-xL (Fig. 5A). An upregulation of survivin and Bcl-xL (to a lesser extent) was also observed in SK-N-SH cells treated with IL-6 (Supplementary Fig. S2C). We then showed that the upregulation of some of these survival proteins was STAT3 dependent by showing that statin inhibited the nuclear expression of survivin in CHLA-255 cells treated with IL-6 and IL-6 + sIL-6R (Fig. 5B) and by documenting a marked decrease in survivin expression and an absence of Bcl-xL expression upon STAT3 knockdown in cells treated with IL-6 and IL-6 plus sIL-6R (Fig. 5C). The data suggested that STAT3-dependent upregulation

of survival factors such as survivin and Bcl-xL was a mechanism by which IL-6 protected neuroblastoma cells from apoptosis.

BMSC and monocytes cooperate to sensitize neuroblastoma cells to IL-6-mediated STAT3 activation

Our *in vitro* data consistently showed that IL-6 was more active on neuroblastoma cells in the presence of sIL-6R. This suggested that sIL-6R sensitized neuroblastoma cells to STAT3 activation by IL-6. To test this hypothesis, we examined whether the addition of sIL-6R at a concentration of 25 ng/mL, which is within the range of the levels detected in the blood of patients with neuroblastoma (10–90 ng/mL; refs. 34, 35), would result in STAT3 activation in the presence of concentrations of IL-6 in the (0.1 to 10) ng/mL range. These experiments showed that when IL-6 was used alone, a concentration of 10 ng/mL was necessary to activate STAT3, whereas when used in combination with sIL-6R (25 ng/mL), a concentration of 0.1 ng/mL of IL-6 was sufficient for STAT3 activation (Fig. 6A). We then tested whether activation of

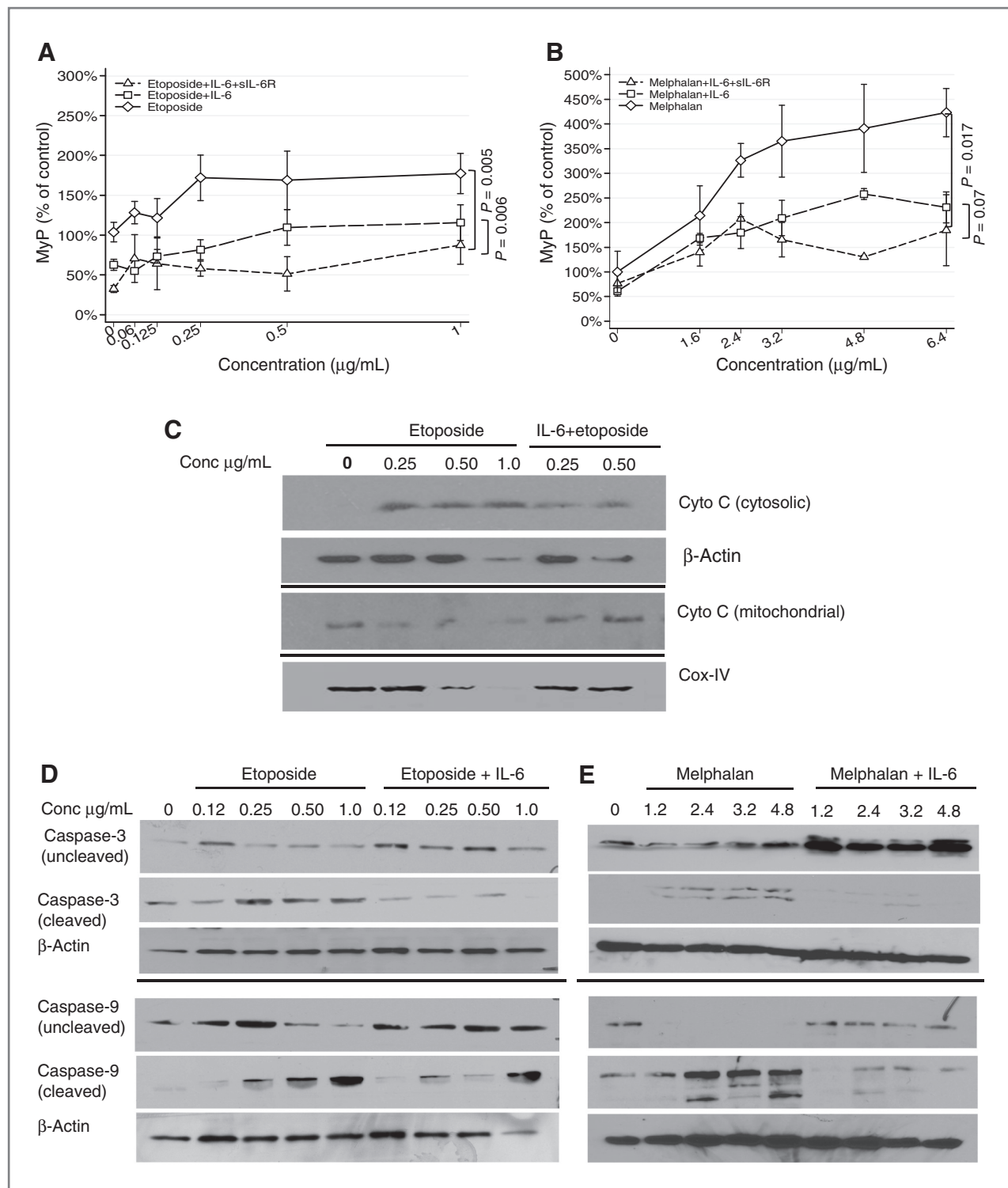


Figure 3. IL-6 and sIL-6R protect neuroblastoma cells from drug-induced apoptosis. A and B, CHLA-255 cells were treated as in Fig. 2. After 24 hours, mitochondrial membrane depolarization (M ψ P) was examined by JC-1 staining by flow cytometry. The data represent the mean (\pm SE) percentage change in depolarization from control cells (untreated with cytotoxic drugs). C, CHLA-255 cells were treated as indicated earlier. After 24 hours, cytosolic and mitochondrial extracts were examined for the presence of cytochrome C by Western blot analysis. The data are representative of 2 separate experiments showing similar results. Cox-IV and β -actin were used as loading control for mitochondrial and cytosolic fractions, respectively. D and E, CHLA-255 cells were treated with IL-6 and etoposide or melphalan. After 24 hours of drug exposure, cell lysates were examined for the expression of full length and cleaved caspase-3 and -9. The data are representative of 2 separate experiments showing similar results. In all gels (C–E), separation lines indicate different gels loaded with the same amount of cell lysate.

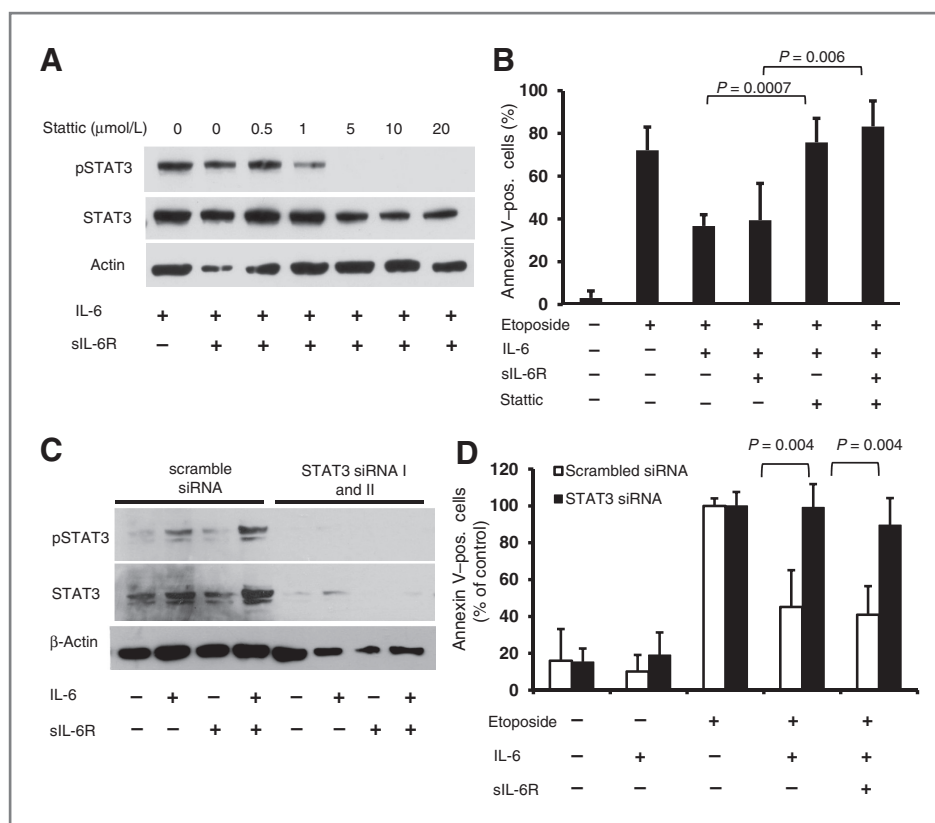
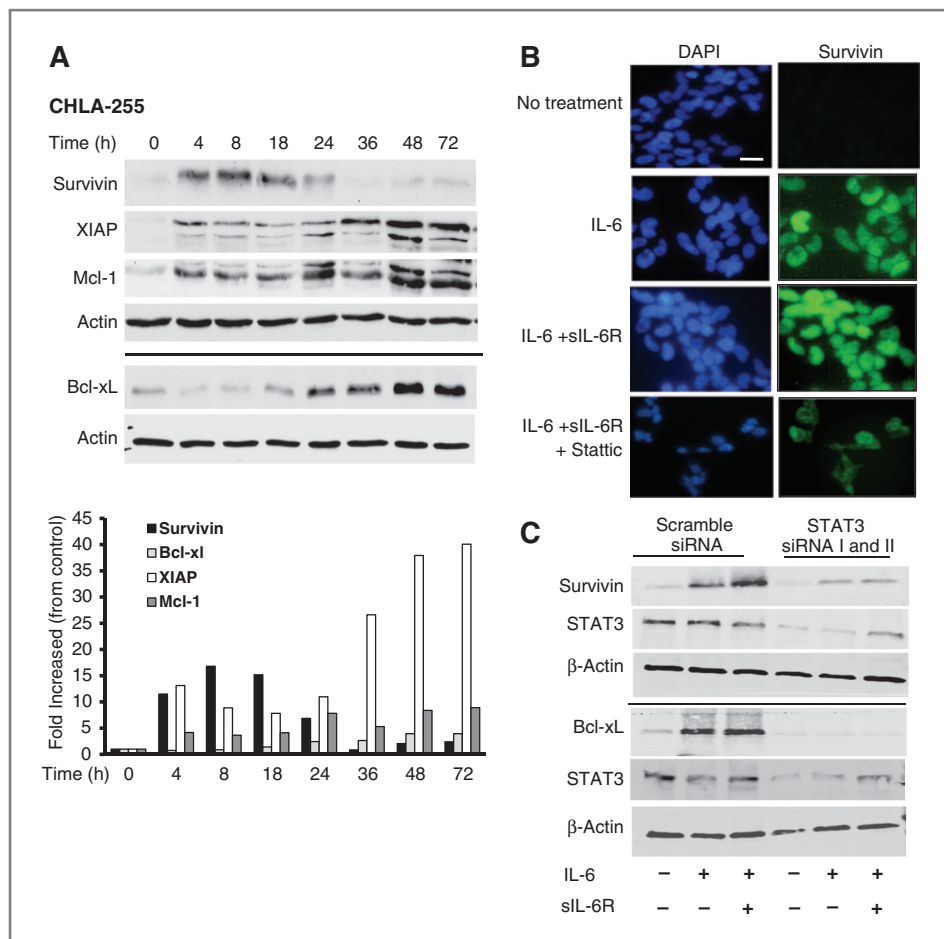


Figure 4. STAT3 is necessary for IL-6-mediated drug resistance. **A**, CHLA-255 cells were treated with IL-6 (10 ng/mL) and sIL-6R (25 ng/mL) for 30 minutes in the presence of static at indicated concentrations. Cell lysates were examined for pSTAT3 and STAT3 expression by Western blot analysis. **B**, CHLA-255 cells were treated with static (2.5 μmol/L) and IL-6 and sIL-6R for 24 hours before being exposed to etoposide (0.25 μg/mL). After 24 hours, cells were examined for Annexin V expression by flow cytometry. The data represent the mean percentage (±SD) of Annexin V-positive cells from 4 samples obtained in 3 independent experiments. **C**, CHLA-255 cells were transfected with STAT3 siRNA or with a scramble sequence. After 72 hours, cells were treated with IL-6 and sIL-6R for 30 minutes and cell lysates were examined for pSTAT3 and STAT3 expression by Western blot analysis. The data are representative of 3 separate experiments showing similar results. **D**, siRNA-transfected CHLA-255 cells were treated with IL-6 and sIL-6R for 24 hours before being exposed to etoposide (0.25 μg/mL) and examined for the presence of Annexin V at the cell surface. The data represent the mean percentage (±SD) of Annexin V-positive cells compared with control (cells treated with etoposide alone) from 4 samples obtained from 3 independent experiments.

STAT3 in the presence of 10 ng/mL of IL-6 could be potentiated by lower concentrations of sIL-6R. The data (Fig. 6B) showed an increase in pSTAT3 activation in the presence of 10 ng/mL of sIL-6R and above but not at lower concentrations (0.1 and 1.0 ng/mL). These sIL-6R concentrations are found in the blood of patients with cancer (35). Because neuroblastoma cells do not produce sIL-6R and are thus dependent of a paracrine source (16), we examined whether it was produced by normal cells in the tumor microenvironment. We first tested BMMSC that we had previously shown to be a source of IL-6 (13). The data showed an anticipated increase in the production of IL-6 (to 1,600 pg/mL) when BMMSC were cocultured with CHLA-255 cells and low amount of sIL-6R (mean 32.5 ± 14.8 pg/mL; Fig. 6C, top). We then tested human monocytes that we had also shown to be a source of IL-6 (14). This experiment revealed that monocytes produced IL-6 and sIL-6R in culture and that their production was significantly increased in the presence of CHLA-255 cells. The amount of sIL-6R produced (mean 149.5 ± 8.6 pg/mL) in the presence of CHLA-255 was

5-fold higher than BMMSC in similar conditions (Fig. 6C, bottom). Although this concentration is lower than the concentration required to enhance STAT3 activation *in vitro* (Fig. 6B), we showed that when human monocytes were cocultured with CHLA-255 cells, STAT3 became activated in the tumor cells even in the presence of low amounts of IL-6 (20 pg/mL) and that activation was in part IL-6R-dependent as it was partially inhibited in the presence of tocilizumab (Fig. 6D). The data thus point to the important contributory function of monocytes to STAT3 activation that is in part mediated by IL-6 and sIL-6R but could involve other activators. We also asked whether STAT3 would be activated in monocytes when cultured in the presence of neuroblastoma cells. In this experiment, CHLA-255 cells and human monocytes were cultured alone or in contact for 24 hours and the mixture of cells was analyzed for the presence of nuclear pSTAT3 and cell surface CD14 (monocytes) and CD56 (neuroblastoma). The data (Fig. 6E and Supplementary Fig. S3B) indicated an absence of pSTAT3 in cells cultured alone but a significant increase in pSTAT3 in both tumor cells and

Figure 5. IL-6 upregulates the expression of antiapoptotic proteins. A, CHLA-255 cells were treated with IL-6 (10 ng/mL) and cells were lysed at indicated time points (hours). Total cell lysates (20 μ g/lane) were then examined by Western blot analysis for the expression of indicated antiapoptotic proteins. Actin was used as loading control. The data are representative of 3 separate experiments showing similar results. The bar graph represents the fold increase compared with control after the ratios of antiapoptotic protein: actin were normalized for the ratio at 0 hour. B, CHLA-255 cells were treated with IL-6 and sIL-6R and examined for survivin expression by immunofluorescence. Stattic (2.5 mmol/L) was added 4 hours before IL-6 and sIL-6R when indicated (scale bar, 20 μ m). C, siRNA-transfected CHLA-255 cells treated as described earlier were examined for STAT3, survivin, and Bcl-xL expression by Western blot analysis after 24 hours of stimulation with IL-6 and sIL-6R. The data are representative of 3 experiments showing similar results.



monocytes after 24 hours of coculture, indicating the presence of a reciprocal STAT3 activation loop between neuroblastoma cells and monocytes.

STAT3 is activated in the bone marrow microenvironment in patients with metastatic neuroblastoma

To provide evidence for a role of STAT3 in patients with metastatic neuroblastoma, we examined STAT3 activation in a series of 10 bone marrow biopsies obtained from children with neuroblastoma. Five specimens were classified as positive for tumor involvement in the marrow (as assessed by the presence of tyrosine hydroxylase-positive cells by immunohistochemistry; ref 36) and 5 were classified as negative for tumor cells. An analysis of the presence of nuclear pSTAT3-positive cells in these samples revealed a higher percentage ($22.3\% \pm 6.06\%$) of nuclear pSTAT3 in samples with tumor cells and a lower percentage ($7.9\% \pm 1.8\%$; $P = 0.002$) in samples negative for tumor cells (Fig. 7A). There was also a higher number of survivin-positive cells in samples infiltrated with tumor cells than in samples without tumor cells (122 ± 69 vs. 10 ± 14 , respectively) and a higher level of Bcl-xL expression (Supplementary Fig. S3C). Because our *in vitro* coculture experiments on tumor cells and monocytes indicated a reciprocal activation of pSTAT3 (Fig. 6D and E), we then asked the question whether

STAT3 was activated in tumor cells and stromal cells in the bone marrow of patients with neuroblastoma. An analysis of pSTAT3 and CD45 by double immunohistochemistry on a bone marrow biopsy sample containing more than 90% tumor cells (Fig. 7B) indicated that 93% of PGP9.5⁺ tumor cells were also pSTAT3-positive and that 42% of CD45⁺ myeloid cells were also positive for pSTAT3. By double immunofluorescence with an anti-CD68 antibody, we then showed the presence of nuclear pSTAT3 in CD68⁺ monocytes/macrophages. The data are consistent with our *in vitro* coculture experiment (Fig. 6E) and indicate that STAT3 in the bone marrow microenvironment is not only activated in tumor cells but also in myeloid cells including monocytes/macrophages (Fig. 7C). To better define subpopulations of these pSTAT3-positive myeloid cells, we examined by flow cytometry an additional 8 fresh bone marrow samples from patients with neuroblastoma for the presence of nuclear pSTAT3 (Supplementary Fig. S4A). Using a combination of 6 surface markers (CD3, CD4, CD14, CD25, CD45, and GD2) and 1 nuclear marker (FoxP3), we identified 6 populations of cells with CD45⁺/GD2⁺ (nonmyeloid), CD45⁺/CD14⁺ (myeloid nonmonocytic), and CD45⁺/CD14⁺ (monocytic) being the most (>10% of total mononuclear cells) abundant (Supplementary Fig. S4B). An analysis of pSTAT3 in these different populations indicated a greater than 10% positive cells in 4 subpopulations,

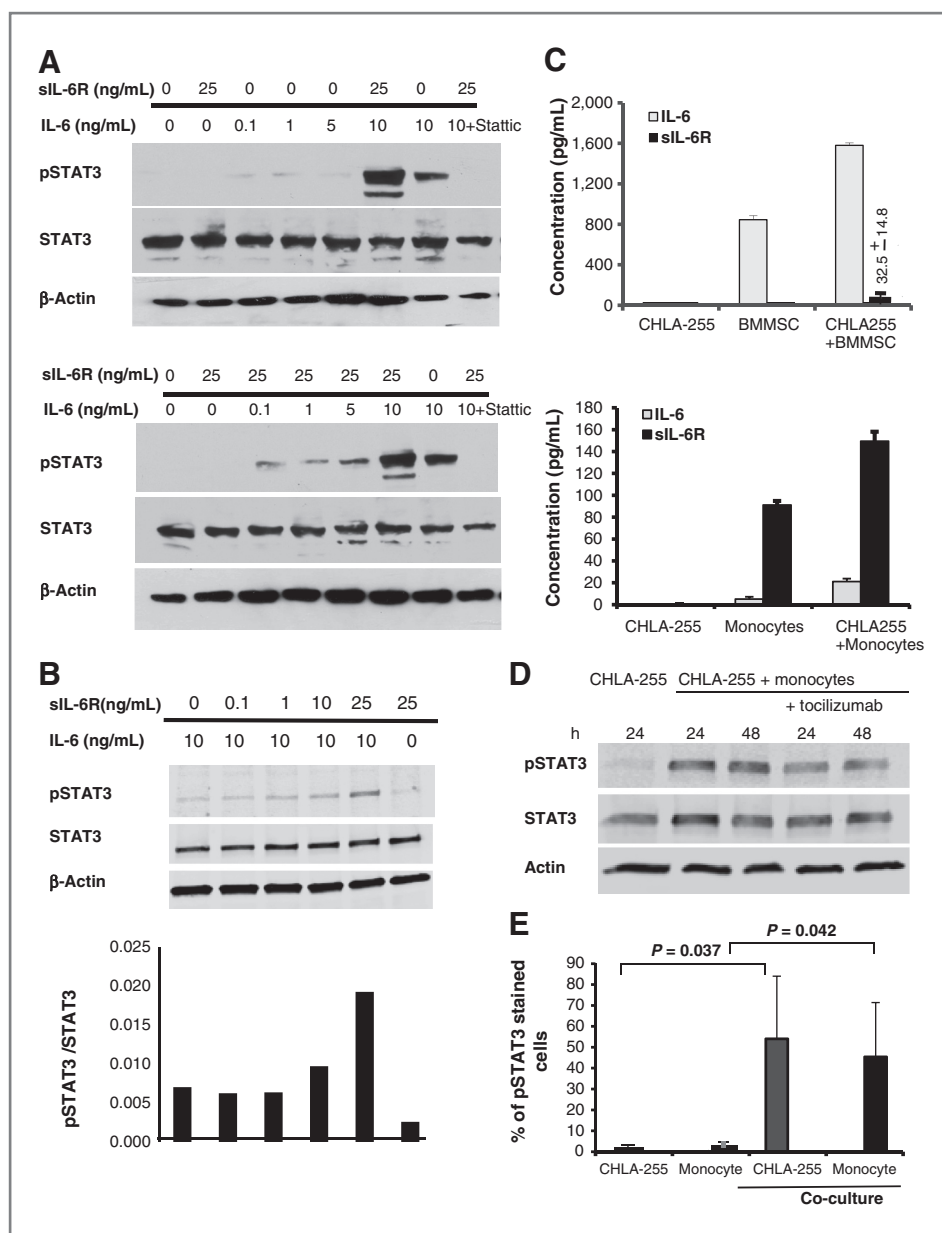
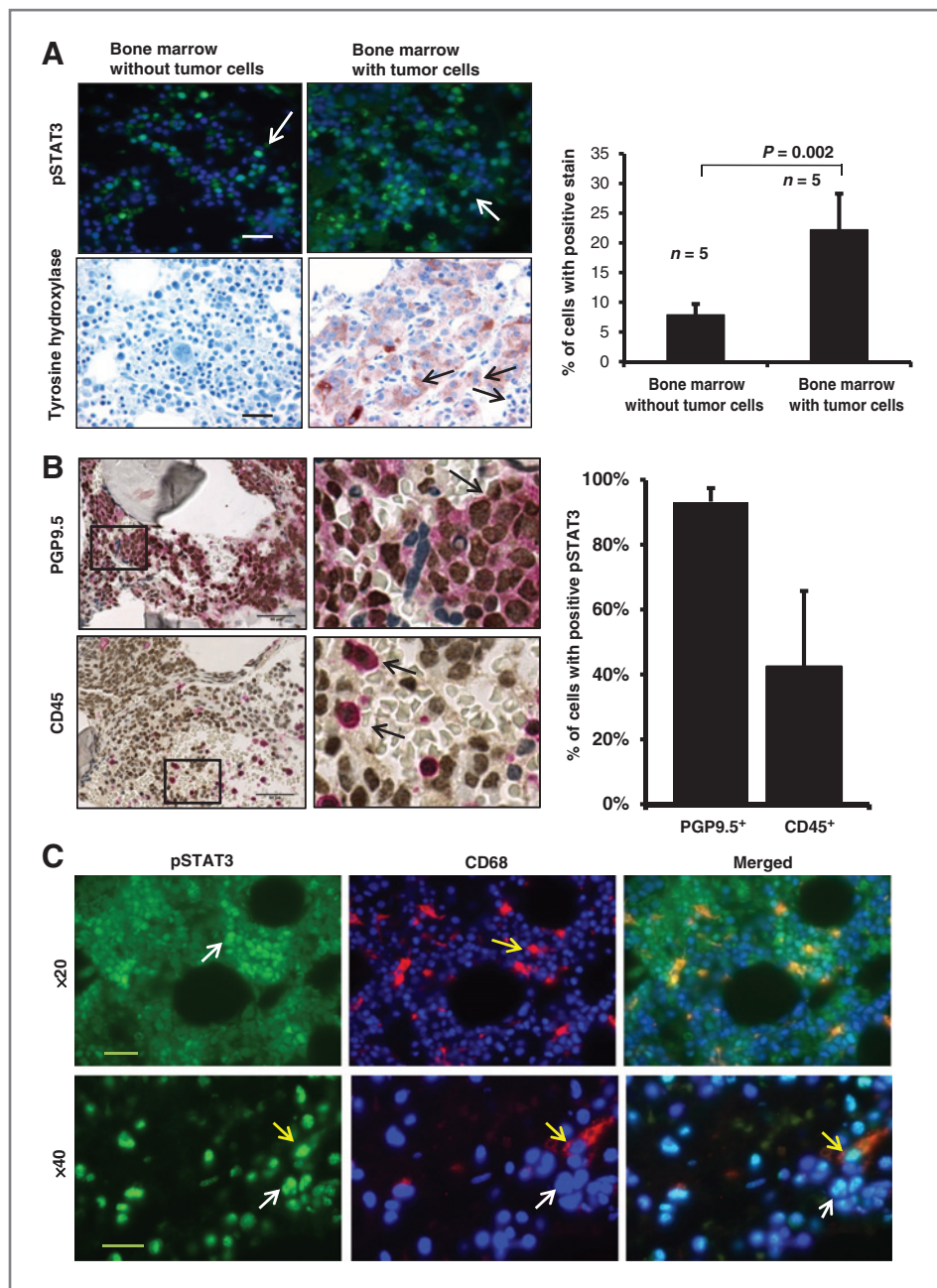


Figure 6. sIL-6R produced by monocytes sensitizes neuroblastoma cells to IL-6-mediated STAT3 activation. **A**, CHLA-255 cells were treated with IL-6 at indicated concentrations in the absence (top) or presence (bottom) of sIL-6R (25 ng/mL) for 30 minutes. The cell lysates were examined for expression of pSTAT3 and STAT3 by Western blot analysis. **B**, CHLA-255 cells were treated with IL-6 alone (10 ng/mL) and in the presence of increased concentrations of sIL-6R (0.1–25 ng/mL) for 30 minutes. The cell lysates were then examined for expression of pSTAT3 and STAT3 by Western blot analysis. The bar diagram represents the ratio pSTAT3/STAT3 obtained by scanning of the blot. **C**, CHLA-255 (4.5×10^5) and human BMMSC or monocytes (4.5×10^5) were cultured either alone or together in Transwell plates for 48 hours. The culture medium was collected and the concentrations of IL-6 and sIL-6R were determined by ELISA. The data represent the mean concentration (\pm SD) of duplicate (top) and triplicate (bottom) samples. **D**, CHLA-255 cells were cocultured with monocytes in Transwell plates in the absence or presence of an anti-IL-6R antibody (tocilizumab). After 48 hours, cell lysates were examined for pSTAT3 and STAT3 by Western blot analysis. **E**, monocytes isolated from the peripheral blood of patients with neuroblastoma and CHLA-255 cells were cultured alone or in contact (ratio tumor cells:monocytes 4:1) for 24 hours. Cells were then harvested and examined by flow cytometry for the presence of nuclear pSTAT3 and expression of cell surface CD56 and CD14 to separate tumor cells from monocytes. The data represent the average percentage of pSTAT3-positive cells (\pm SD) from 3 separate samples.

CD45⁺/GD2⁺ nonmyeloid, nontumor cells, CD45⁺/GD2⁺ tumor cells, CD45⁺/CD14⁺ monocytes, and CD45⁺/CD3⁺/CD4⁺/CD25⁺/FoxP3⁺ regulatory T cells (Treg), indicating that in addition to monocytes, Treg and nonmyeloid cells

could be part of the reciprocal loop of STAT3 activation between tumor cells and bone marrow-derived cells. Thus our data identify an IL-6/sIL-6R/STAT3 interactive pathway between neuroblastoma cells and the tumor

Figure 7. STAT3 is activated in the bone marrow microenvironment in patients with metastatic neuroblastoma. **A**, left, paraffin-embedded sections of bone marrow biopsies from patients ($n = 10$) with neuroblastoma were examined by immunofluorescence for the presence of nuclear pSTAT3 (white arrow) and by immunohistochemistry for the presence of tyrosine hydroxylase-positive tumor cells (black arrow; bar, 50 μm). Right, the data represent the mean percentage ($\pm\text{SD}$) of positive nuclei determined in 5 high magnification ($\times 40$) fields per sample. **B**, bone marrow biopsy infiltrated with more than 90% of neuroblastoma cells was examined by dual immunohistochemistry as described in Materials and Methods for the presence of pSTAT3 in PGP9.5⁺ tumor cells (top) or CD45⁺ myeloid cells (bottom; arrows indicate dual positive cells). The histogram on the right side represents the mean ($\pm\text{SD}$) percentage of PGP9.5⁺ and CD45⁺ cells that were also positive for pSTAT3 from 5 high-power field areas (scale bar, 50 μm). **C**, the presence of pSTAT3-positive and CD68⁺ cells in bone marrow samples was examined by dual immunofluorescence (white arrows indicate nuclear localization of pSTAT3 in tumor cells and yellow arrows in CD68⁺; scale bar, 50 μm at $\times 20$ and 20 μm at $\times 40$).



microenvironment that contributes to drug resistance and in which STAT3 has a necessary function.

Discussion

Overexpression of IL-6 by BMMSC has a dual protumorigenic function on neuroblastoma cells by promoting osteoclast activation (13) and tumor cell proliferation and survival (16). Here, we show the presence of an IL-6/sIL-6R/STAT3 paracrine pathway of interaction between tumor cells and the microenvironment that provides tumor cells with the ability to counteract the apoptotic effect of cytotoxic agents. Constitutive activation of STAT3 by oncogenes

such as Src, Fes, Sis, PyMT, Ros, or Eyk has been reported (37) but not by MYC-N, which is often amplified in neuroblastoma (38). Among the 8 cell lines examined, one, SK-N-BE (2), exhibited amplification of the MYC-N oncogene and in this cell line—as in most other cell lines without MYC-N amplification—STAT3 was not constitutively active. Our data indicate that in contrast to many other types of cancer, STAT3 activation is rarely constitutive in neuroblastoma but seems dependent on the tumor microenvironment. Although our data specifically point to a role for IL-6 as activator of STAT3, they do not rule out the possibility that other cytokines and growth factors could also contribute.

Our data identify a central function for the IL-6/sIL-6R/STAT3 pathway in conferring drug resistance to neuroblastoma cells. This effect involves the upregulation of several antiapoptotic proteins in particular survivin, Bcl-xL, Mcl-1, and XIAP that are known transcriptional targets of STAT3 (25, 39, 40). The observation that survivin is downstream of STAT3 signaling is relevant as high levels of survivin in neuroblastoma have also been associated with poorer clinical outcome (41). It has been previously reported that IL-6-mediated STAT3 activation plays a role in EMDR in myeloma (42), however, the mechanisms involved seem different between the 2 types of cancer. In myeloma, activation of STAT3 by IL-6 is primarily mediated by β 1 integrin-dependent adhesion of myeloma cells to bone marrow stromal cells (8). In contrast, in neuroblastoma, the expression of IL-6 in the bone marrow stroma is primarily regulated by soluble factors produced by tumor cells including prostaglandin E2 (16) and galectin-3-binding protein (43) among others.

Our data provide a new insight into the contributory role of monocytes to STAT3 activation by showing that monocytes produce sIL-6R and that sIL-6R enhances the sensitivity of neuroblastoma cells to IL-6-mediated STAT3 activation. The importance of sIL-6R in IL-6/gp130 signaling in autoimmunity, inflammation and cancer has been recently emphasized (44). Elevated levels of sIL-6R have been reported in the blood of patients with cancer including neuroblastoma and myeloma, and are typically a marker of unfavorable clinical outcome (34, 35, 45). These blood levels (ng/mL) are higher by 100-fold than the *in vitro* concentration of sIL-6R observed in cocultures of neuroblastoma cells and human monocytes. Although the reason for this discrepancy is not entirely clear, our data show a strong activation of STAT3 in cocultures of neuroblastoma cells and monocytes that is in part suppressed by a blocking antibody against IL-6R supporting a potentiating effect also at lower concentrations in coculture when IL-6 and sIL-6R are constantly produced and secreted. The data nevertheless do not rule out the possibility of other interactive pathways of activation. For example, a reciprocal activation of STAT3 between tumor cells and bone marrow-derived cells has recently been described and shown to be mediated by the sphingosine-1 phosphate receptor 1 (S1PR1), a member of the G protein-coupled receptor family of the lysophospholipids that sustains STAT3 activation by IL-6 (46). Whether S1PR1 plays such a role in sustaining STAT3 activation in neuroblastoma is presently investigated by our laboratories. Our data point to the important role that monocytes could play in EMDR in cancer, a new function recently showed in a mouse model of mammary carcinoma by Denardo and colleagues, who showed that suppression of macrophage infiltration in tumors increases response to chemotherapy (47). A similar protective role of monocytes in cis-platinum-induced apoptosis in colon and lung cancer-initiating cells has also been recently reported (48).

Interestingly, our analysis of pSTAT3 expression in bone marrow samples of patients with neuroblastoma revealed the presence of pSTAT3 not only in tumor cells and in CD45⁺ myeloid cells including CD68⁺ monocytes/macrophages, but also in a CD25⁺, FoxP3⁺ Treg cells. Activation of pSTAT3 in

Treg cells has been shown to be mediated by IL-23 produced by TAM under STAT3 activation, which leads to the expression of FoxP3 and the secretion of the immunosuppressive cytokines IL-10 by Treg (49). Finally, we also observed a subpopulation of CD45⁺/GD2⁺ bone marrow cells that expressed pSTAT3. Although these cells were not fully characterized at this point, they could represent in part mesenchymal cells that we have shown to play a critical intermediary role in bone invasion in neuroblastoma (13).

Ultimately, our data raise the question whether inhibition of IL-6-mediated STAT3 activation in neuroblastoma could play a role in therapy by preventing EMDR. Several humanized mAbs against soluble and membrane bound IL-6R (tocilizumab, REGN88) or against IL-6 (siltuximab and sirukumab) are currently in clinical trials for Castleman's disease, rheumatoid arthritis, and several types of cancer (44). Other potentially active agents include small-molecule inhibitors of JAK, some of them being currently tested in clinical trials (50, 51). Our data suggest that in neuroblastoma, these inhibitors may be most effective in combination with cytotoxic agents to prevent EMDR.

In summary, we provide here a new mechanistic insight on the contributory role of the bone marrow microenvironment in promoting drug resistance in neuroblastoma, as we show that by being a source of IL-6 and sIL-6R, the bone marrow microenvironment provides tumor cells with the ability to resist the cytotoxic effects of chemotherapy through STAT3 activation.

Disclosure of Potential Conflicts of Interest

Y.A. DeClerck is a consultant/advisory board member of AACR/Editorial Board. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Ara, M.A. Sheard, H. Shimada, R. Buettner, S.G. Groshen, L. Ji, R.C. Seeger, Y.A. DeClerck

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Ara, R. Buettner

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Acknowledgments

The authors thank J. Rosenberg for her excellent assistance in preparing the article.

Grant support

The work was supported by an NIH grant PO1 CA 84103 (R.C. Seeger and Y.A. DeClerck), a NIH grant U54 CA163117 (Y.A. DeClerck), and a ThinkCure grant (R.C. Seeger). T. Ara was the recipient of a grant from the Children's Cancer Research Fund and the Children's Neuroblastoma Cancer Foundation.

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Received June 18, 2012; revised March 20, 2013; accepted April 3, 2013; published OnlineFirst April 30, 2013.

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